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We believe that the domestic laying hen has great potential as an animal model for ovarian cancer prevention research. Unlike other ovarian cancer models, which require experimental induction of ovarian tumors, chickens develop ovarian adenocarcinoma spontaneously, with an incidence ranging from 13 to 40 percent between four and six years of life. No investigators have taken advantage of the chicken to study ovarian cancer. Thus, the chicken ovarian cancer model has yet to be validated and developed. As part of a prevention trial in chickens funded by the Department of Defense, we have accumulated 1400 chicken reproductive tracts including 140 with adenocarcinomas, and gathered valuable data regarding the natural history of these tumors. This provides the remarkable opportunity to critically evaluate the chicken ovarian cancer animal model and determine its relevance to human ovarian cancer research. The aim of the current proposal is to increase our understanding of the molecular and histologic features of chicken ovarian cancers. In addition, we will develop a histologic classification for chicken ovarian cancers, which is a critically important prerequisite to the widespread use of this animal model for ovarian cancer research.

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Introduction

Epithelial ovarian cancer is a highly lethal malignancy. It is the fifth leading cause of cancer deaths among women in the United States and causes 140,000 deaths annually in women worldwide. Despite intensive research efforts over the past decade directed towards improved detection and treatment, the long-term survival of women with ovarian cancer has only improved modestly. Progress in the fight against ovarian cancer has been hampered by a number of factors, including late diagnosis, molecular heterogeneity of tumors, absence of highly curative chemotherapy, and lack of a valid animal model.

We believe that development of effective chemopreventive agents for ovarian cancer represents our best hope for decreasing ovarian cancer mortality in the future. Based on our studies in primates and in the laboratory, we are convinced that the well-known protective effect of oral contraceptives against ovarian cancer is due, in large part, to the molecular biologic effects of progestins on the ovary. We have found that progestins activate the apoptosis pathway in the ovarian epithelium, making it more likely that cells that have incurred genetic damage will be eliminated, rather than develop into cancer. A number of other apoptosis-inducing agents also hold promise for preventing ovarian cancer, including retinoids. Ultimately, it is our goal to develop a preventive strategy using the best chemopreventive agents, either alone or in combination, in order to achieve maximum protection against ovarian cancer.

At this time, the lack of a valid ovarian cancer animal model is a major obstacle to ovarian cancer prevention research. In order to develop pharmacologic preventive strategies for ovarian cancer in a timely fashion, animal models that closely mimic human ovarian cancer are desperately needed. Human prevention trials are costly requiring large numbers of subjects and many years to complete. Development of an animal model for ovarian cancer prevention research would represent a significant breakthrough and lead to expedited evaluation of numerous candidate agents. Ideally, this would lead to rapid identification of a select number of agents, which have the greatest potential for ovarian cancer prevention and that can then be evaluated in human prevention trials.

We believe that the domestic laying chicken has great potential as an animal model for studying chemoprevention of ovarian cancer. Unlike other animal models for ovarian cancer, which generally require the experimental induction of ovarian tumors, the chicken develops ovarian cancer spontaneously. The domestic hen is the only animal with a high incidence of spontaneous ovarian adenocarcinoma, ranging from 13 to 40 percent between four and six years of life. No investigators have taken advantage of the chicken to study ovarian cancer. Thus, the chicken ovarian cancer model has yet to be validated and developed. As part of a chemoprevention grant awarded to us by the Department of Defense in 1998, we have performed a two-year chemoprevention trial in the chicken designed to test the hypothesis that progestins confer chemopreventive effects against ovarian cancer. We are now conducting a second prevention trial in the chicken, funded by the NIH, evaluating progestin and the retinoid 4-HPR as candidate preventives.

We have accumulated 140 chicken reproductive tract cancers, including 98 ovarian tumors, from our first prevention trial, and gathered valuable data regarding the

natural history of these tumors. In addition, we are gathering both tumor and natural history data from our second prevention trial that is underway. Data and tissues that we are gathering provide us with the remarkable opportunity of being able to critically evaluate the chicken ovarian cancer animal model and determine its relevance to human ovarian cancer research. The aim of the current proposal is to increase our understanding of the molecular and histologic features of chicken ovarian cancers. In addition, we will develop a histologic classification for chicken ovarian cancers, which is a critically important prerequisite to the widespread use of this animal model for ovarian cancer research. For this proposal, we plan to characterize and develop the chicken ovarian cancer model by (1) analyzing the molecular and genetic features of chicken ovarian cancers, including alterations in the p53 tumor suppressor gene and the Her-2/neu and Ras oncogenes, (2) classifying the morphologic and histologic features of chicken ovarian cancers, leading to the development of a histologic classification for chicken ovarian adenocarcinomas, and (3) comparing the molecular and histologic features of ovarian cancers that develop in chickens receiving synthetic progestins compared to untreated controls. We hope to gather evidence that chicken ovarian cancers have genetic alterations and morphologic features similar to those identified in human ovarian carcinomas, thereby validating the chicken ovarian cancer model.

BODY:

Aim 1: To determine whether the genetic alterations that characterize ovarian cancers in women are also a feature of ovarian cancers in the domestic fowl.

The genetic characterization of the chicken reproductive tract cancers is well underway. Over 100 frozen tumor samples have undergone extraction of DNA and RNA. In addition, sections have been cut and mounted on charged slides for immunohistochemical analysis. Primers have been designed for both p53 and H-Ras. Thus far, reverse transcriptase PCR has been used to amplify the p53 gene. The entire coding region has been screened for alterations using single stranded conformational analysis and direct sequencing of variant bands has been performed. Clearly deleterious p53 mutations have been found in approximately 14% chicken ovarian cancers. The majority of these (93%) are insertions deletions that predict truncated protein products. There was one missense mutation that results in an amino acid substitution. All the mutations were located between amino acids 101 and 336, which correspond to the DNA binding domains. Four unique silent single nucleotide polymorphisms were detected in 9 of the cancers. An identical 22-base pair insertion polymorphism in the 3-prime untranslated region of the gene was identified in 14 samples. Over the next year, we plan to screen oviductal tumors for p53 mutations. In addition, in birds that have gross tumors in both the oviduct and ovary, we will screen for p53 mutations at both sites to determine whether the tumors are similar (derived from one precursor) or distinct.

Primers have been designed for H and K-Ras, and sequencing is now underway to determine the incidence and location of mutations in these genes.

The optimal staining methods for Her-2-neu are currently being worked up in preparation for staining for Her 2-neu.

As the studies outlined in Aim #2 are completed, the pattern of gene expression and mutation will be compared among tumors from birds subjected to different preventive treatments to see if treatment characteristics influence the genotype of reproductive tract cancers in the chicken.

Aim 2: To classify the morphologic and histologic features of chicken ovarian cancers, leading to the development of a histologic classification of chicken ovarian adenocarcinomas.

Significant progress has been made in the histologic evaluation and classification of reproductive tract tumors accrued from the chemoprevention study funded by the Department of Defense (DAMD 17-98-1-8686) under our prior Duke Program Project grant. Reproductive tracts have been obtained and processed from 1400 of the 1405 four-year-old laying hens at the termination of the prevention trial. Gross lesions of most birds with tumors and other reproductive tract pathology have been photographed. Samples from each tract have been collected, fixed for 72-hours in 10% buffered neutral formalin, and transferred to 70% alcohol. Additional samples of tumors and normal tissue (kidney) from selected birds were snap frozen at the time of necropsy for subsequent genomic analysis. Slides have been prepared from all birds with grossly evident tumors or suspect lesions found when the tissues were trimmed. Slides have been prepared and stained with hematoxylin and eosin following conventional paraffin embedding and sectioning for initial characterization. To provide a 'gold standard' for the accuracy of gross tumor identification, tissues from all birds in two of the 6 groups (n = 274) in the study and approximately 10% of birds with normal appearing reproductive tracts randomly selected from each of the remaining four groups will be examined for tumors. Additional tissues are being collected from suitable birds in a chemoprevention study involving 2277 birds that is currently ongoing. Immunostaining with cytokeratin (AE1/AE3) and ovalbumin (to detect oviductal glandular epithelial characteristics) was done previously on a small group of tumors.

Dr. John Barnes (DVM, Avian Pathologist) and Dr. Stanley Robboy (MD, Gynecologic Pathologist) have been performing a meticulous examination of chicken reproductive tract cancers from our prevention trials. Within the chicken reproductive tract, adenocarcinomas occur in both the ovary and or oviduct with nearly equal frequency. More birds have cancers in both the ovary and oviduct than at either site alone. Among 140 reproductive cancers that have been identified to date, 38 reside only in the ovary, 42 reside only in the oviduct, and 60 reside in both the ovary and oviduct. Hens with reproductive tract adenocarcinomas often have moderate to marked ascites and widespread to generalized coelomic implantation (carcinomatosis). When extensive, implantation and local invasion of the duodenum and pancreas causes fibrosis and distortion of tissues. Unless the tumor is advanced or there is concurrent peritonitis, the birds are generally in good condition. Some hens continue to ovulate from unaffected areas of the ovary and produce eggs.

Tumors in the ovary are firm to hard, multilobular, irregular, solid or pedunculated, and tan to cream colored. They frequently have a cauliflower-like appearance. Some nodules may be umbilicated. Cysts containing clear, yellow, red, or

green fluid are often present. Adenocarcinoma of the oviduct typically occurs as thickened, firm areas with irregular lobular patterns in the oviduct wall. Occasionally nodules extend either extra- or intraluminally but, on microscopic examination, mural oviductal tumors do not breach the mucosal epithelium.

Microscopically, adenocarcinomas in the ovary, oviduct, or both reproductive tissues are similar. The basic pattern is lobules or nodules composed of short tubules or spherules that are lined with a secretory, simple, cuboidal, or low columnar epithelium and usually contain eosinophilic proteinaceous fluid in their lumen. They have variable amounts of interstitial fibrovascular tissue and/or smooth muscle bundles, which accounts for their scirrhous nature grossly. Squamous differentiation, osseous metaplasia, and cystic and/or papillary patterns occur infrequently. Often tumors can be observed grossly on the surface of ova, but microscopically they are in perifollicular spaces and do not penetrate the follicle wall. Mitosis is uncommon to rare except in more anaplastic areas where cells may be found in sheets rather than tubules.

The histology is variable and not distinct between ovarian and oviductal adenocarcinomas both among birds and at times within the same bird or even a single tumor. They can be divided into: 1) tubular, secretory, cytoplasmic granules present (well differentiated); 2) tubular, secretory, cytoplasmic granules not found (less differentiated); 3) tubular/follicular, secretory, squamous differentiation, 4) anaplastic. Except for poorly differentiated and anaplastic adenocarcinomas, it is generally possible to find at least a few cells that have cytoplasmic droplets characteristic of those in the oviductal glands. However, it should not be inferred that they necessarily arise in the oviduct and spread to the ovary. A number of ovarian adenocarcinomas have been obtained in which a careful gross and microscopic examination of the oviduct failed to reveal any evidence of neoplasia, but the characteristics of the tumor cells in the ovary are those of oviductal glandular epithelium. Our findings indicate these are true ovarian tumors composed of oviductal-like cells, much like the cells of adenocarcinomas of woman can be composed of cells with characteristics of oviductal (serous), uterine (endometrioid), cervical (mucous), or bladder (transitional) epithelium. Dissimilarity of cell morphology between chicken and human ovarian tumors can be attributed to anatomical and functional differences. The mammalian reproductive tract provides an environment for fetal development whereas the avian reproductive tract is primarily secretory. Further, our findings suggest that tumors involving both oviduct and a significant portion (> 25%) of the ovary with cancer in the medulla along with cortical tumors, likely represent a multicentric origin of the tumors (common for avian neoplasia) rather than spread from the oviduct to the ovary as the literature suggests. Nodules confined to the cortex and comprising < 25% of the ovary are not distinct from similar implants on serous surfaces in the body cavity and are considered to be secondary to a primary tumor elsewhere (usually oviduct).

Cells that give rise to reproductive tract adenocarcinomas, especially those in the ovary, in chickens are currently unknown. Tumor cells stain positive for cytokeratin, which indicates they are epithelial in origin, but cytokeratins that can differentiate various epithelia in birds have not been identified yet. Cells with cytoplasmic granules typical of those in the albumin-secreting mucosal glands of the oviduct magnum stain positively for ovalbumin suggesting an oviductal origin or differentiation. These cells may originate and differentiate from the germinal epithelium covering the ovary, small embryonic rests

of oviductal cells embedded in the ovary, rete ovarii, or another site not currently anticipated.

In the next 12 months, we will conduct a detailed histologic examination of the different tumors and examine selected ones by immunohistochemistry, especially for characteristics of oviductal glandular epithelium.

Key research accomplishments

- Basic techniques established
- Tumor collection that will form the basis for studies established
- Initial characterization of reproductive tract tumors identifying morphologic affinities between well-differentiated tumor cells and normal cells of oviduct glandular epithelium
- Established the likely relationship between oviduct and ovarian cancers and proposed the theory of a multicentric origin of tumors
- Developed criteria for identifying different tumor types
- Validation of methods underway

This project is in its early developmental stage. The results of these studies will mature and be reported in the future.

Reportable outcomes

1) An abstract has been submitted to the Society of Gynecologic Oncologists for presentation of the p53 sequencing work in chicken tumors. The annual meeting of the Society is scheduled for March of 2002.
2) Funding was applied for and granted by the NCI Prevention branch for a prevention trial in the chicken, evaluating the candidate preventives 4-HPR (a retinoids derivative) and levonorgestrel (a progestin). The trial is scheduled to last two years; the primary outcome measure will be the incidence of reproductive tract tumors. In addition, tumors collected during the trial will be analyzed for p53, Ras, and Her-2 neu, similar to what is planned for the current grant funded by the DOD. The work funded by the NCI will thus provide additional chicken reproductive tract tumor specimens for molecular and analysis, and also allow us to examine whether tumors that arise in birds on different types of hormonal treatment have different molecular phenotypes.
3) We have applied for funding from the Department of Defense under the Duke Program Project Renewal (7/2001) for studies that will include an avian prevention trial in the chicken, evaluating the preventive efficacy of various progestin dosages and schedules, with or without the addition of Vitamin D, on the outcome measure of reproductive tract tumors in the chicken.
4) An application has been submitted to the RAPID program within the Prevention Branch at the NCI for a broad research plan for development of ovarian cancer preventive agents. The plan includes avian prevention trials in the chicken.

Conclusions

The availability of a valid ovarian cancer animal model, especially one in which cancers develop spontaneously at a high rate, would represent a critically important breakthrough for ovarian cancer prevention research. An animal which develops spontaneous ovarian cancer would be ideal for ovarian cancer prevention studies, and provide the means through which a large variety of preventive agents can be quickly evaluated, thereby expediting the development of promising chemopreventive agents that could subsequently be tested in human prevention trials. The demonstration that ovarian cancers in the fowl are similar to those seen in women would be a critical step towards establishing the validity of the chicken model for testing chemopreventive agents. Whereas chemoprevention trials for ovarian cancer in women are difficult because of the relatively low annual incidence of the disease, chickens have a high incidence over a relatively short life span. Demonstration of efficacy of an ovarian cancer chemopreventive strategy in chickens would provide a credible rationale and enthusiasm for testing a similar strategy in women.

Our efforts in developing the chicken ovarian cancer model will help overcome a major obstacle in ovarian cancer prevention research, and provide a means for the rapid development of effective preventives for ovarian cancer.

References

Barnes HJ, Rodriguez GC, Carver DK. Reproductive tract tumors in mature laying hens. Proc. 138th Ann Conv Am Assoc Vet Med, Boston, MA, July 14-18, 2001. (http://www.cvm.ncsu.edu/info/departs/fae/PHM/RTTMLH/intro.html)

Appendices

Preliminary results from previous chemopreventative study. Tumors for this work were obtained from these birds. Note that inducing anovulation decreased the occurrence of reproductive tract adenocarcinomas. Treatment of anovulatory birds with progestins and/or vitamin D resulted in a further reduction in the number of birds with cancer.